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A Low Molecular Mass Heat-Shock Protein Is Localized to Higher Plant Mitochondria

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When pea (*Pisum sativum* L. var Douce Provence) plants are shifted from a normal growth temperature of 25°C up to 40°C for 3 h, a novel 22-kD protein is produced and accumulates in the matrix compartment of green leaf mitochondria. HSP22 was purified and used as antigen to prepare guinea pig antiserum. The expression of HSP22 was studied using immunodetection methods. HSP22 is a nuclear-encoded protein de novo synthesized in heat-stressed pea plants. The heat-shock response is rapid and can be detected as early as 30 min after the temperature is raised. On the other hand, HSP22 declines very slowly after pea leaves have been transferred back to 25°C. After 100 h at 25°C, the heat-shock pattern was undetectable. The precise localization of HSP22 was investigated and we demonstrated that HSP22 was found only in mitochondria, where it represents 1 to 2% of total matrix proteins. However, the induction of HSP22 does not seem to be tissue specific, since the protein was detected in green or etiolated pea leaves as well as in pea roots. Finally, examination of matrix extracts by nondenaturing polyacrylamide gel electrophoresis and immunoblotting with anti-HSP22 serum revealed a high-molecular mass heat-shock protein complex of 230 kD, which contains HSP22.

Induction of HSPs in response to high-temperature stress is a universal phenomenon. HSPs exhibit highly ubiquitous and conserved features from bacteria to humans, and they are proposed to be essential for cell survival (Lindquist and Craig, 1988). In higher plants, the low molecular mass HSP class (15–30 kD) is preponderant. Low molecular mass HSPs have been identified in many diverse species, among both dicots and monocots (Kloppstech et al., 1985; Vierling et al., 1986, 1989). Four gene families encoding low molecular mass HSPs have been distinguished in plants (for review, see Vierling, 1991). The gene products consist of highly conserved HSPs that were detected in three different cell compartments: the endomembrane system (Helm et al., 1993), cytosol, and chloroplasts (for review, see Vierling, 1991). Surprisingly, limited information is available on the biochemical characterization of low molecular mass HSPs in plant mitochondria (Lin et al., 1984; Chou et al., 1989; Hartman et al., 1992). Therefore, it was of interest to determine whether plant mitochondria accumulate low molecular mass HSPs during heat stress. In the present paper, we characterize a low molecular mass protein of 22 kD (HSP22) that is nuclear encoded and localized in the matrix of mitochondria isolated from heat-stressed pea (*Pisum sativum* L.) leaves (pea plants treated at 40°C for 3 h). We also report the accumulation rate of HSP22 during the stress period and its stability with time after the heat exposure.

MATERIALS AND METHODS

Plant Material, Growth, and Heat-Shock Conditions

Plants were grown from mature pea seeds (*Pisum sativum* L. var Douce Provence) in vermiculite for 14 d at 25°C, either under a 12-h photoperiod of white light from fluorescent tubes (500 μmol m^{-2} s^{-2}) or in the dark for etiolated plants. Plants were watered every day with tap water.

Heat stress was administrated by incubating light-grown or etiolated 14-d-old intact plants at 40°C for 3 h. Growth chamber (Bioclim, Weiss Technik, Lindenstruh, Germany, 1600 sp) temperature was increased from 25°C to 40°C in 30 min. A RH of 70% was maintained throughout the stress period to minimize plant dehydration. Leaves and roots were harvested immediately after the stress period.

Mitochondria and Matrix Protein Isolation

Mitochondria were isolated and purified from approximately 3 kg of fully expanded leaves of control pea plants or heat-shock-treated pea plants. The method was described by Douce et al. (1987), using self-generating Percoll gradients and a linear gradient of 0 to 10% (w/v) PVP-25 (Serva) (top to bottom). Pea root mitochondria and etiolated pea leaf mitochondria isolations were performed from approximately 1 kg and 500 g, respectively, of material, as described by Douce et al. (1987), using a simple self-generating gradient. The mitochondria (at least 95% intact as judged by their impermeability to Cyt *c* [Douce et al., 1972]) were subsequently concentrated by differential centrifugation. The amount of extramitochondrial contamination in the Percoll-purified mitochondrial fraction was determined by measuring the activity of various marker enzymes for cytosol (pyrophosphate:Fru-6-P-1-phosphotransferase, EC 2.7.1.90), chloroplasts (glyceraldehyde-3-P-dehydrogenase, EC 1.2.1.13), and peroxisomes (catalase, EC 1.11.1.6; hydroxypyruvate reductase, EC 1.1.1.81) (Lunn et al., 1990). This analysis showed that extramitochondrial contamination was negligible (results not shown).

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Abbreviation: HSP, heat-shock protein.

Isolated mitochondria (about 100 mg of protein) were lysed by 50-fold dilution in a buffer containing 5 mM Tris-HCl, pH 7.0. After vortexing, the mitochondria were frozen and then thawed three times in liquid N_2 to ensure complete lysis (Bourguignon et al., 1988). Membranes were removed by centrifugation at 100,000*g* for 1 h (36,000 rpm in a Beckman SW-40 rotor). The resulting supernatant fraction (matrix proteins) was concentrated through a PM 10 membrane (Filtron, Northborough, MA) (final protein concentration about 20 mg/mL). The membrane pellet was rinsed and resuspended in the lysis buffer with deoxycholate 0.4% (final protein concentration about 10 mg/mL).

Chloroplasts and Stroma Protein Isolation

Chloroplasts were isolated and purified from green pea leaves according to the method described by Walker et al. (1987) using discontinuous Percoll gradients. Isolated chloroplasts were treated with the same procedure as mitochondria to separate the stroma and the membrane proteins.

Total Protein Extraction

Total protein extracts were prepared following the method of Hurkman and Tanaka (1986) with slight modifications. Tissues were ground to a fine powder in liquid N_2 with a mortar and a pestle. Powder (500 μg) was adjusted in a microfuge tube to 600 μL with 0.7 M Suc, 0.5 M Tris-HCl, pH 8, 5 mM EDTA, 0.1 M NaCl, 300 mM 2-mercaptoethanol, and 2 mM PMSF. Phenol (1 volume) was added and the mixture was vigorously shaken at room temperature for several minutes. The two phases were separated by centrifugation and the upper phenol phase was collected. Proteins were precipitated from the phenol phase with 5 volumes of 0.1 M ammonium acetate in methanol at −20°C. After centrifugation, the pellet was washed three times with methanolic 0.1 M ammonium acetate and once with acetone. The proteins were solubilized in 50 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromphenol blue, and 10% glycerol, and stored at −20°C.

Gel Electrophoresis

Electrophoresis was performed at room temperature in SDS-polyacrylamide slab gels containing a 7.5 to 15% (w/v) linear polyacrylamide gradient and proteins were separated in the buffer system described by Laemmli (1970).

One-dimensional gel electrophoresis of matrix proteins under native conditions was carried out without SDS on a linear acrylamide gradient (3.5–27%) with a 3.5% acrylamide stacking gel, as described by Clarke and Critchley (1992). Electrophoresis was performed at a constant current of 20 mA for 24 h. Under these conditions, proteins and protein complexes migrate until reaching their gel pore exclusion limit (Clarke and Critchley, 1992; Helm et al., 1993).

Two-dimensional gel electrophoresis was performed as described by O'Farrell (1975). In the first dimension, proteins were separated by IEF from pH 3.5 to 9. Separation in the second dimension was carried out on a 7.5 to 15% polyacrylamide gel in denaturing conditions (SDS-PAGE). Gels were stained with Coomassie brilliant blue.

Partial Purification of HSP22 and Antiserum Preparation

Matrix proteins (approximately 25 mg of protein) were loaded on a Superdex-200 gel filtration (120 mL, Pharmacia) column previously equilibrated in medium A (20 mM KH_2PO_4, 1 mM DTT, 1 mM EDTA, 400 mM KCl, pH 7.2). After sample loading, the column was eluted with medium A at a flow rate of 0.5 mL min^{-1} and 1-mL fractions were collected. Since HSP22 had no known activity, purification was followed by gel electrophoresis under denaturing conditions of aliquots from each collected fraction. Fractions containing HSP22 were pooled, concentrated, and dialyzed against medium B (medium A without KCl). The pooled fractions were layered on a Mono-Q HR 5/5 (Pharmacia) column equilibrated with medium B. Proteins were eluted off the Mono-Q column by a 0 to 500 mM KCl gradient in medium B, with a flow rate of 0.5 mL min^{-1}. Aliquots of collected fractions were analyzed as described above. All purification steps were performed at 4°C. Fractions containing HSP22 were precipitated with 10% (v/v) TCA and loaded on a 7.5 to 15% SDS-PAGE system. HSP22 bands were excised from the Coomassie blue-stained gel, ground to a powder in liquid N_2, and injected as an emulsion with Freund's adjuvant into a guinea pig for raising polyclonal anti-HSP22 antibodies.

Immunoblotting Analysis

After separation by PAGE, the proteins were electrophoretically transferred onto nitrocellulose membranes (Bio-Rad) using a Bio-Rad electrotransfer apparatus. Following a wash in medium C (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 30 g/L [w/v] BSA) for 1 h, membranes were first incubated with guinea pig antiserum against HSP22 in medium C overnight at 4°C and then washed in medium C and incubated with goat anti-guinea pig IgG, alkaline phosphatase conjugated (Sigma, 1:10,000 diluted in medium C). Membranes were stained for alkaline phosphatase activity. The color reaction was revealed by incubating membranes in 0.1 mM Tris, pH 9.5, 4 mM $MgCl_2$, nitroblue tetrazolium chloride (0.1 mg/mL), and 5-bromo-4-chloro-3-indolyl-phosphate (0.06 mg/mL).

RESULTS

Characterization and Partial Purification of HSP22 from Pea Leaf Mitochondria

Fourteen-day-old pea plants were heat stressed at 40°C for 3 h in 70% RH. Mitochondria were isolated and treated to analyze soluble and membrane proteins on a 7.5 to 15% polyacrylamide gel under denaturing conditions (SDS-PAGE). Coomassie blue staining shows that heat treatment (40°C for 3 h) induced accumulation of a novel protein of apparent molecular mass 22 kD in the matrix (Fig. 1A, lane 2). In contrast, this polypeptide was not present in the control (25°C) matrix (Fig. 1A, lane 1) or in membrane protein extracts (Fig. 1A, lane 3, control [25°C], and lane 4, heat treatment [40°C for 3 h]). By scanning the Coomassie blue-stained polypeptides (scanner Shimadzu CS 9000), we estimated that HSP22 represents roughly 1 to 2% of the total

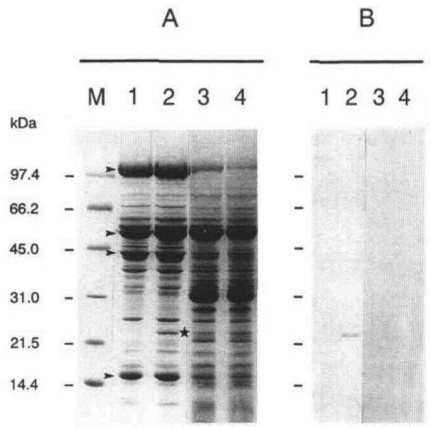

Figure 1. Analysis of mitochondrial proteins extracted and purified from control (25°C) and heat-stressed pea leaves (40°C, 3 h). A, One-dimensional SDS-PAGE, Coomassie blue stained; B, western blot analysis after incubation with HSP22 antibodies. M, Molecular mass markers (kD); lanes 1, control matrix proteins (40 μg); lanes 2, heat-stressed matrix proteins (40 μg); lanes 3, control membrane proteins (40 μg); lanes 4, heat-stressed membrane proteins (40 μg). The star indicates the novel HSP22, induced by the heat treatment (3 h at 40°C). The arrows to 97, 45, and 15.5 kD indicate three subunits of the Gly decarboxylase complex, the P-protein, the T-protein, and the H-protein, respectively. The arrowhead to 53 kD indicates the subunit of the homotetramer Ser hydroxymethyltransferase. The antiserum dilution is 1:40,000 in medium C containing 30 g/L BSA (see "Materials and Methods").

matrix proteins. Two-dimensional gel electrophoresis was also performed on matrix proteins. When comparing patterns of heat-stressed pea leaf mitochondria to control, it is easy to visualize the 22-kD protein as a unique acidic form, with an isoelectric point around 6 (Fig. 2, A, control [25°C], and B, heat treatment [40°C for 3 h]).

HSP22 was purified from the matrix extract using liquid chromatography methods. At each step, eluted fractions were analyzed by one-dimensional SDS-PAGE stained with Coomassie blue to allow HSP22 detection. Control (25°C) and heat-stressed (40°C for 3 h) materials were treated in parallel. Heat-stressed matrix proteins (25 mg) were fractionated by gel filtration on the Superdex-200 column and HSP22 eluted just after the void volume in the 150- to 250-kD molecular mass range (HSP22 emerged from the column between the standards catalase [240 kD] and aldolase [160 kD]). Fractions containing HSP22 were pooled (5 mg of protein), concentrated, and dialyzed against medium B before loading on a Mono-Q column (see "Materials and Methods"). HSP22 was eluted off the Mono-Q column at 260 mM KCl. This behavior demonstrates the relative negative charge of HSP22 at pH 7.2 and confirms the acidic isoelectric point of the polypeptide determined by two-dimensional electrophoresis. Fractions eluted from the Mono-Q column that contained HSP22 (800 μg of protein) were submitted to electrophoresis on a 7.5 to 15% SDS-PAGE and the band corresponding to HSP22 was excised. The amount of purified HSP22 was estimated to be around 25 μg. The purification procedure yielded about 25 μg of HSP22, starting from 25 mg of matrix proteins, with a 10% recovery assuming that HSP22 represents about 1% of the total matrix proteins. It must be remembered that preparation of the mitochondrial matrix already represents an important purification of HSP22. Therefore, the actual purification of HSP22 from crude leaf extract is more than 600-fold. The HSP22 bands were excised from SDS-PAGE to prepare guinea pig antiserum.

Expression of HSP22 by Immunoblotting Experiments

The anti-HSP22 serum was first tested on a mitochondrial matrix extract. Figure 1B indicates that the HSP22 band was present in the matrix extract obtained from heat-stressed (40°C for 3 h) pea plants (lane 2), whereas no immunoreaction was detectable in matrix extract obtained from control (25°C) pea plants (lane 1). Likewise, no reaction was observed with mitochondrial membranes in heat-stressed material (Fig. 1B, lane 4) or in control material (Fig. 1B, lane 3). These results confirm our previous observations on Coomassie blue-stained gels (see Fig. 1A).

We have confirmed the internalization of the HSP22 within the mitochondria isolated from heat-shocked pea plants. The

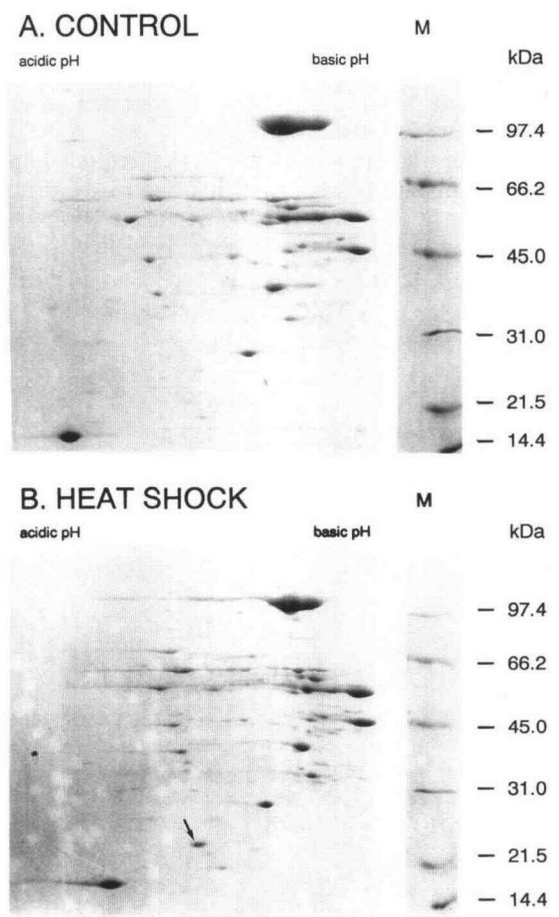

Figure 2. Two-dimensional electrophoretic analysis of soluble mitochondrial proteins extracted from leaves of control pea plants (180 μg of protein), grown at 25°C (A) or from pea plants heat stressed at 40°C for 3 h (180 μg of protein) (B). M, Molecular mass markers (kD). The pH range is from 3.5 to 8.5. The arrow indicates the novel HSP22 induced by high-temperature treatment.

control was carried out in the presence of thermolysin (Boehringer), a Ca^{2+}-dependent protease (Fig. 3). Pea leaf mitochondria were extracted and purified from heat-shocked whole pea plants at 40°C for 3 h. Intact isolated mitochondria (15 mg of protein) exhibiting a high degree of integrity (97%) were incubated with gentle shaking at 4°C for 1 h in buffer 1 (0.3 M mannitol, 10 mM KH_2PO_4, [pH 7.2]) in the presence of 200 μg/mL thermolysin and 1 mM $CaCl_2$. After a 1-h incubation at 4°C, addition of 10 mM EGTA stopped the reaction. Intact mitochondria were then purified again on a Percoll cushion (10% in buffer 1). Controls were treated in the same way, using intact mitochondria (8 mg of protein) in the presence of 200 μg/mL thermolysin, 1 mM $CaCl_2$, and 10 mM EGTA to inhibit thermolysin activity. Matrix proteins were isolated from both mitochondrial samples. At the same time, we have submitted matrix proteins from mitochondria of heat-shocked pea plants to the protease activity to make sure that HSP22 was sensitive to thermolysin. Matrix proteins were separated by electrophoresis on a polyacrylamide gel under denaturing conditions. HSP22 was immunodetected by western blot. Figure 3 shows that HSP22 is sensitive to the protease treatment when thermolysin is added to isolated matrix proteins (lane 4), whereas the stress protein is protected from proteolysis when thermolysin is added to intact mitochondria (lane 2). We concluded that HSP22 is internalized within the mitochondria and is protected from the protease by mitochondrial membranes.

The effect of translation inhibitors was then tested on pea leaves, cut from light-grown pea plants, and placed in 20 mM KH_2PO_4 buffer (pH 6.5) with the addition of chloramphenicol or cycloheximide at 100 μg/mL, prior to being stressed at 40°C for 3 h. Total protein extracts were then analyzed by SDS-PAGE and immunoblotting. Figure 4 indicates that cycloheximide but not chloramphenicol inhibited the expression of HSP22, thus demonstrating that HSP22 is nuclear encoded.

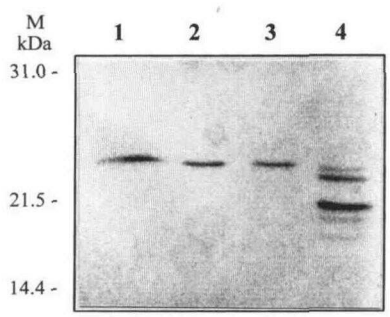

Figure 3. HSP22 sensitivity to thermolysin added to intact mitochondria or isolated matrix proteins. Matrix proteins were isolated and separated on one-dimensional polyacrylamide gels under denaturing conditions. Western blot analysis was done after incubation with anti-HSP22 serum (dilution 1:40,000 in medium C containing 30 g/L BSA). M, Molecular mass markers (kD). Lane 1, Matrix proteins from heat-shocked pea leaf mitochondria (whole pea plants treated at 40°C for 3 h). Lane 2, Matrix proteins from heat-shocked pea leaf mitochondria. Intact purified pea leaf mitochondria were previously treated with 200 μg/mL thermolysin and 1 mM $CaCl_2$. Lane 3, Matrix proteins from heat-shocked pea leaf mitochondria. Intact purified pea leaf mitochondria were previously treated with 200 μg/mL thermolysin, 1 mM $CaCl_2$, and 10 mM EGTA. Lane 4, Matrix proteins from heat-shocked pea leaf mitochondria treated with 200 μg/mL thermolysin and 1 mM $CaCl_2$.

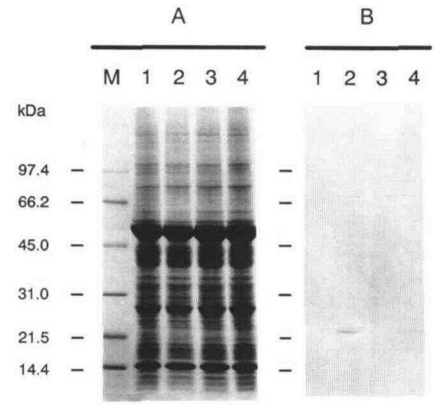

Figure 4. Effect of translation inhibitors on HSP22 synthesis. A, Total protein extracts of control or heat-stressed pea plants were prepared and separated on SDS-PAGE (Coomassie blue stained). B, Western blot analysis after incubation with HSP22 antibodies. M, Molecular mass markers (kD). Control (25°C, lanes 1) or high-temperature treatments (40°C for 3 h, lanes 2–4). The antiserum dilution is 1:5000 in medium C containing 30 g/L BSA (see "Materials and Methods"). During the heat treatment, leaves cut from normal pea plants were placed in Pi buffer (20 mM KH_2PO_4, pH 6.5) (lanes 1 and 2), with the addition of cycloheximide (lanes 3) or chloramphenicol (lanes 4) at 100 μg/mL.

Finally, to visualize the time-dependent accumulation of HSP22, total pea leaf proteins were prepared during the heat-stress period and analyzed by SDS-PAGE and immunoblotting. Figure 5 indicates that the heat-shock response is rapid: HSP22 can be detected in total protein extracts as early as 30 min after the beginning of the stress period and reached its highest level after 3 h at 40°C. Figure 5 also shows the effect of shifting heat-stressed pea leaves back to 25°C. Under these conditions, HSP22 abundance was maintained for 3 d but was barely detectable or even absent by 96 h.

Localization of HSP22

We have purified intact chloroplasts from control and heat-stressed pea plants. Stromal extracts were analyzed by SDS-PAGE and immunoblotting; no 22-kD band was observed, demonstrating that HSP22 did not accumulate in the chloroplastic compartment (not shown), further supporting the mitochondrial localization of HSP22.

To investigate whether HSP22 was synthesized in non-photosynthetic tissues, we have purified mitochondria from etiolated pea leaves and from pea roots. Figure 6 shows the Coomassie blue-stained SDS-PAGE analysis of matrix proteins from the two isolates, for control (lanes 1 and 7) and for heat-stressed plants (lanes 2 and 8). Western blot analysis with anti-HSP22 serum showed the presence of HSP22 in both heat-stressed samples (lanes 4 and 6). On the other hand, and as previously shown in green material, HSP22 was not constitutively expressed in control tissues (lanes 3

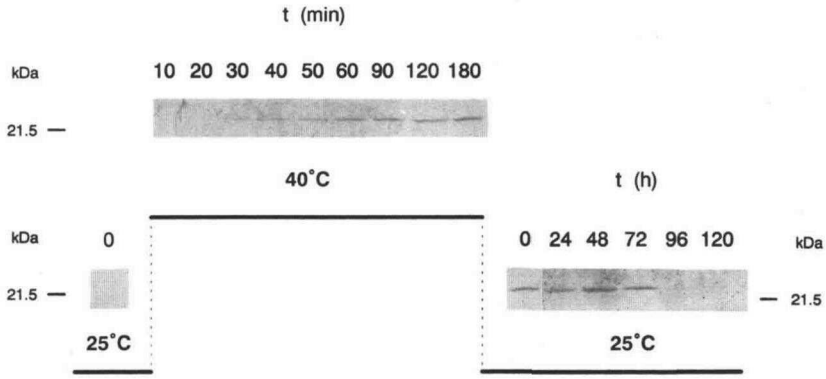

Figure 5. Time-course HSP22 accumulation. Total protein extracts from control or heat-stressed pea plants were separated by SDS-PAGE and then analyzed by western blot after incubation with HSP22 antibodies (dilution 1:5000 in medium C with 30 g/L BSA). Upper western blot, HSP22 accumulation during a 3-h heat shock (40°C). Lower western blot, HSP22 disappearance after shifting heat-stressed pea plants back to the control temperature (25°C)

and 5). Thus, it is clear that HSP22 accumulated in photosynthetic and nonphotosynthetic organs, including roots and etiolated leaves.

Moreover, roots were cut from normal pea plants and placed in a 20-mM KH_2PO_4 buffer (pH 6.5) prior to being stressed at 40°C for 3 h, as realized for isolated pea leaves. Total protein extracts were then analyzed by SDS-PAGE and immunoblotting. HSP22 was immunodetected in heat-stressed, excised roots. Under the same conditions, we also tested the effect of the two translation inhibitors, cycloheximide and chloramphenicol, on HSP22 expression. As shown in leaves, HSP22 accumulated in heat-stressed, excised pea roots treated with chloramphenicol. Cycloheximide, in contrast, prevented HSP22 synthesis (not shown). These results confirm that HSP22 is a nuclear-encoded protein and also indicate that HSP22 is not imported in roots from leaves via the phloem.

Complex Organization of HSP22 in Native Conditions

The apparent native molecular mass of HSP22 was estimated by one-dimensional electrophoresis of matrix proteins extracted from heat-stressed pea plants without denaturing agents (Fig. 7). The Coomassie blue-stained gel is shown in Figure 7, lane 1. Immunoblot analysis of the native gel after transfer to nitrocellulose membrane revealed an immunoreactive band in the heat-stressed extract with an apparent molecular mass of 230 kD (Fig. 7, lane 2).

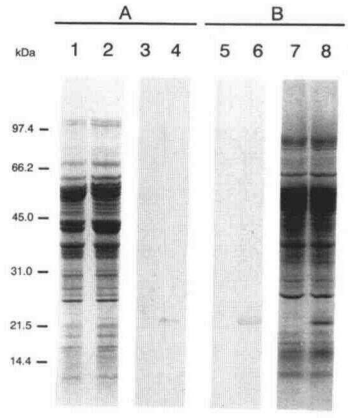

Figure 6. Analysis of soluble mitochondrial proteins extracted from pea roots (A) and from etiolated pea leaves (B). Lanes 1 and 2, Coomassie blue-stained SDS-PAGE of mitochondrial matrix proteins from control (lane 1) or heat-stressed (lane 2) pea roots. Lanes 3 and 4, Western blot analysis of mitochondrial matrix proteins from control (lane 3) or heat-stressed (lane 4) pea roots, after incubation with HSP22 antibodies. Lanes 5 and 6, Western blot analysis of mitochondrial matrix proteins from control (lane 5) or heat-stressed (lane 6) etiolated pea leaves, after incubation with HSP22 antibodies. Lanes 7 and 8, Coomassie blue-stained SDS-PAGE of mitochondrial matrix proteins from control (lane 7) or heat-stressed (lane 8) etiolated pea leaves. Molecular mass markers are indicated on the left (kD). The antiserum dilution is 1:10,000 in medium C with 30 g/L BSA.

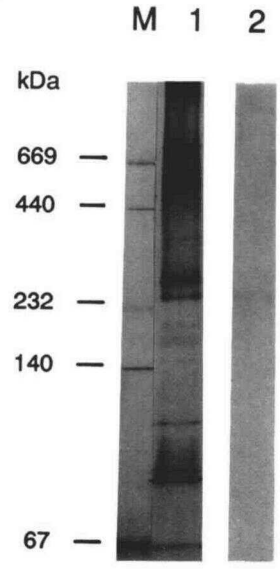

Figure 7. Separation and immunological analysis of native matrix proteins (200 μg) from pea leaf mitochondria extracted from heat-stressed pea plants (40°C for 3 h). Lane 1, One-dimensional PAGE, without any denaturing agent, Coomassie blue stained. Lane 2, Western blot analysis, after incubation with HSP22 antibodies (dilution 1:10,000 in medium C with 30 g/L BSA). High molecular mass markers (kD) are indicated on the left (M). The immunodetected band revealed by the HSP22 antiserum corresponds to a 230-kD particle.

DISCUSSION

The results reported in this article indicate that the accumulation of a novel 22-kD polypeptide (HSP22) in pea leaf mitochondria is induced by high temperatures. We have demonstrated that HSP22 was internalized within heat-stressed pea leaf mitochondria and was not a contaminating protein from the cytoplasm or from other organelles. HSP22 was found in the matrix space and was not associated with the inner mitochondrial membrane. The heat-stress conditions used in our experiments are close to those found in nature, since 40°C is near the upper limit of temperatures reached by plant leaves during summer. Under heat-shock conditions, the expression of nuclear-encoded HSP22 is induced, yielding final concentrations of up to 1 to 2% of the total matrix proteins. Therefore, HSP22 belongs to the class of HSPs that are not constitutive but are synthesized de novo in response to stress (Mansfield and Key, 1987). Such a result was not attributable to bacterial contamination, as suggested by Nieto-Sotelo and Ho (1987) for mitochondria and plastids isolated from maize tissues, because cycloheximide but not chloramphenicol inhibited the expression of HSP22 (Fig. 5).

It is now well established that plants produce a specific subset of HSPs in response to elevated temperatures, among which the complex group of low molecular mass proteins (15–30 kD) predominates (Mansfield and Key, 1987; Vierling, 1991). The HSP22 localized to pea leaf mitochondria could, according to its molecular mass, be considered a member of this family. Most of these HSPs were localized in the chloroplast stroma from various plants including bean (Süss et al., 1986), tobacco (Neumann et al., 1989), barley (Clarke and Critchley, 1992), maize (Vierling et al., 1986; Nieto-Sotelo et al., 1990), and soybean (Vierling et al., 1989). Surprisingly, HSP21 from pea leaf chloroplasts was found either in stroma (Vierling et al., 1986) or bound to thylakoid membranes (Kloppstecht et al., 1985). Some of these low molecular mass HSPs may be found in mitochondria (Lin et al., 1984; Cooper and Ho, 1987; Chou et al., 1989; Hartman et al., 1992). In the case of barley (Hartman et al., 1992) the induction pattern of mitochondria was reported to be complex, revealing that heat shock enhanced synthesis of four constitutive polypeptides of apparent molecular masses of 72, 60, 24, and 10 kD. On the other hand, analysis of proteins from soybean seedlings (Chou et al., 1989) revealed the presence of heat-shock-inducible proteins with apparent molecular masses of 15 to 18 kD, and two distinct polypeptides of 22 and 24 kD. In this case, the polypeptides of apparent molecular mass 15 to 18 kD accumulate in the cytosol and become associated with cell organelles including mitochondria in response to thermal stress. All these HSPs are thought to somehow protect as well as facilitate recovery from the adverse changes in the cell's environmental circumstances. However, the mechanism by which low molecular mass HSPs may effect plant protection has not been determined.

Since HSP22 represents 1 to 2% of the total matrix proteins, this polypeptide level is important relative to other matrix components, including tricarboxylic acid cycle enzymes and protein components of the Gly decarboxylase complex. The situation is comparable with cytoplasmic low molecular mass HSPs, which are abundant, but is in contrast with HSP21 in pea chloroplasts (Chen et al., 1990). Such a relative abundance suggests that HSP22 plays an essential role within mitochondria from heat-stressed pea plants. Since HSP22 is also present in roots or etiolated leaves, we strongly suggest that this protein plays a structural or protective role of the matrix components, whereas chloroplastic HSP21 may have a catalytic role in stroma (Chen et al., 1990). HSP22, for example, could help in the stabilization of a fragile multienzymic complex involving tricarboxylic cycle enzymes (metabolon) (Robinson and Srere, 1985). It is also possible, as shown recently for several low molecular mass HSPs that exhibit chaperon capacities (Jakob et al., 1993), that HSP22 may have a function in the formation or maintenance of the native conformation of polypeptides. It is clear that the long half-life of this protein and its rapid synthesis support the hypothesis that HSP22 is involved in establishing thermotolerance at the level of the mitochondria.

Finally, the results presented in this article indicate that HSP22 localized in pea mitochondria was present in an oligomeric structure corresponding to a molecular mass of approximately 220 to 230 kD when matrix preparations were examined by denaturing PAGE. It is also possible that HSP22 is associated with a specific matrix protein. This alternative interpretation is particularly attractive in light of the potential function of low molecular mass HSPs being a molecular chaperon (Jakob et al., 1993). In fact, the participation of low molecular mass HSPs in multimeric structures is a common feature in all organisms (Nover and Scharf, 1984), and it is very likely that a highly conserved region in the amino acid sequence of these HSPs facilitate their aggregation state. However, barley chloroplast HSP24, the presumed HSP22 homolog of the chloroplast, did not aggregate into a multimeric structure in vivo (Clarke and Critchley, 1992). This observation suggests that barley chloroplast HSP24 does not have a homologous function to mitochondria HSP22. With the use of the purified HSP22 as well as our polyclonal antibody, studies are in progress to further characterize the structure and function of this interesting small HSP.

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